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(54) Title: ANNEXIN DERIVATIVES WITH ENDOGENOUS CHELATION SITES

(57) Abstract: Modified annexins having an N-terminal chelation site comprising an amino acid extension are disclosed. The amino acid extension includes an amino acid sequence X<sub>1</sub>-Gly-X<sub>2</sub>, where X<sub>1</sub> and X<sub>2</sub> are selected from Gly and Cys, where at least one of X<sub>1</sub> or X<sub>2</sub> is Cys. Radionuclides are readily chelated by the modified annexins to provide radiolabeled annexins useful in imaging vascular thrombi and apoptosis *in vivo*.

## ANNEXIN DERIVATIVES WITH ENDOGENOUS CHELATION SITES

### Field of the Invention

The present invention relates to annexin derivatives having chelation sites, radiolabeled annexin derivatives, and imaging methods using radiolabeled annexin derivatives.

### Background of the Invention

The formation of vascular thrombi is a significant complicating factor for atherosclerosis and coronary trauma or disease. Techniques for determining the presence of vascular thrombi include invasive techniques that are often cumbersome and fail to detect thrombi with good sensitivity and specificity. Available non-invasive techniques are often of limited value and fail to image arterial thrombi. Common arterial thrombosis imaging use radionuclide methods that are complex and time consuming, and limited in their practical utility. More importantly, these methods generally fail to detect small thrombi which are of significant clinical importance, particularly in coronary arterial thrombi.

Activated platelets associated with vascular thrombi, express phosphatidylserine, an anionic phospholipid, in an amount significantly greater than quiescent platelets, which express little, if any, phosphatidylserine. Annexins are a class of proteins that are characterized by calcium-mediated binding to anionic phospholipids. Annexin V is a human protein of 319 amino acids with a molecular weight of 36,000 Daltons and binds to phosphatidylserine with a high affinity ( $K_d = 7$  nM/L). Accordingly, annexin V offers the potential for selective targeting of platelet thrombi. Furthermore, because there is virtually no circulating annexin V endogenous pool to compete for binding sites on thrombi or to dilute exogenously

administered annexin V, annexin V is an attractive candidate for the non-invasive detection of vascular thrombi. Recently, radiolabeled annexins derivatives have been used to image vascular thrombi *in vivo*. Stratton et al., Circulation, 92:3113-3121, 1995.

5 In addition to its association with vascular thrombi, cell surface expression of phosphatidylserine also occurs during apoptosis. One of the earliest events in programmed cell death is the externalization of phosphatidylserine, a membrane phospholipid normally restricted to the inner leaflet of the lipid bilayer. Cells undergoing apoptosis redistribute phosphatidylserine from the inner leaflet of the  
10 plasma membrane lipid bilayer to the outer leaflet.

Cell death can occur either through necrosis, which results in uncontrolled release of a variety of intracellular substances, or through apoptosis, which is an orchestrated sequence leaving little cellular residue. Through apoptosis, cellular debris is absorbed by neighboring cells without damage to adjacent tissue or  
15 extracellular matrix. Apoptosis (or programmed cell death) plays an important role in a number of physiological events including embryogenesis, regulation of the immune system, and homeostasis. Programmed cell death also plays a role in the pathogenesis of a number of disorders including AIDS and other viral illnesses, cerebral and myocardio ischemia, autoimmune and neurodegenerative diseases, organ  
20 and bone marrow transplant rejection, and tumor response to chemotherapy and radiation.

Apoptosis has been determined in histological sections with *in situ* staining of DNA breaks, formed by the cleavage of chromatin by endonucleases, by terminal deoxynucleotidyl-transferase-mediated deoxyuridine triphosphate-biotin nick-and  
25 labeling imaging.

Because annexin has a high affinity for cell membranes expressing phosphatidylserine, annexin V derivatives have been utilized to detect apoptosis in hematopoietic cells, neurons, fibroblasts, endothelial cells, smooth muscle cells, carcinomas, lymphomas, all murine embryonic cell types and plant and insect cells.  
30 The utility of a radiolabeled annexin V for *in vivo* imaging of phosphatidylserine expression associated with apoptosis has been reported. Blankenberg et al., Proc. Natl. Acad. Sci. U.S.A., 95:6349-6354, 1998. In the report, phosphatidylserine expression during programmed cell death was detected and imaged through the use of a radiolabeled annexin conjugate ( $^{99m}\text{Tc}$  HYNIC-annexin V). The use of the same  
35 radiolabeled annexin conjugate for *in vivo* imaging of apoptosis during cardiac

allograft rejection has also been reported. Vriens et al., The Journal of Thoracic and Cardiovascular Surgery, 116:844-853, 1998.

Despite the benefits and advantages related to the annexin-based imaging agents noted above, a need for improved annexin-based imaging agents that are effective as imaging vascular thrombi and apoptosis and more readily prepared than the currently used annexin conjugates. The present invention seeks to fulfill these needs and provides further related advantages.

#### Summary of the Invention

In one aspect, the present invention provides a modified annexin having an N-terminal chelation site. By virtue of the chelation site, the modified annexin readily chelates a radionuclide to provide a radiolabeled annexin. In a preferred embodiment, the modified annexin includes an endogenous chelation site that is formed recombinantly. Methods for forming the modified annexin and radiolabeled annexin are also provided.

In another aspect of the present invention, a method for imaging vascular thrombi is provided. In the method, vascular thrombi are imaged using a modified annexin having an N-terminal chelation site to which is complexed a radionuclide.

In a further aspect, the present invention provides a method for imaging apoptosis. In the method, apoptosis is imaged using a modified annexin having an N-terminal chelation site to which is complexed a radionuclide.

#### Brief Description of the Drawings

The foregoing aspects and many of the attendant advantages of this invention will become more readily appreciated by reference to the following detailed description, when taken in conjunction with the accompanying drawings, wherein:

FIGURES 1A-1C are chemical structures for the chelation sites of the invention shown with chelated technetium; and

FIGURE 2 schematically represents pET-12a-PAP1.

#### Detailed Description of the Preferred Embodiment

The present invention relates to annexin derivatives. As used herein, the term "annexin" refers to a class of proteins characterized by their ability to bind with high affinity to membrane lipids in the presence of millimolar concentrations of calcium. Annexins have been shown to exhibit anticoagulatory effects that are mediated by the binding of annexins to negatively charged surface phospholipids (e.g., on activated platelets). Annexin V is a representative annexin molecule used in the description of the present invention. Annexins within the scope of the invention include

annexins I, II, III, IV, V, VI, VII, VIII, XI, XIII, XXXI, and XXXII. The term “annexin” includes native annexin purified from natural sources such as, for example, human placenta, or annexin molecules containing a native sequence produced through genetic engineering, recombinant, or other means. The term  
5 “annexin” includes modified annexins as defined below, derived from or produced by any source. As used herein, the term “modified annexin” refers to an annexin molecule wherein the native sequence or molecule is altered in such a way without materially altering the membrane binding affinity of the annexin. Such annexins can be produced by chemical, genetic engineering, or recombinant techniques. The  
10 modification can include sequence modification through the addition of several amino acid residues, and/or an addition/deletion of an amino acid at a single site on the native or genetically engineered sequence. In the context of the present invention, modified annexins include annexins modified at the N-terminus by the addition of amino acid residues.

15 In one aspect, the present invention provides a modified annexin suitable for radiolabeling with a diagnostic imaging agent. In a preferred embodiment, the present invention provides a modified annexin having an N-terminal chelation site comprising an amino acid extension. The amino acid extension includes an amino acid sequence having at least one glycine and at least one cysteine in the amino acid  
20 sequence. In a preferred embodiment, the amino acid extension has an amino acid sequence  $X_1\text{-Gly-X}_2$ , where  $X_1$  and  $X_2$  are selected from Gly and Cys, where at least one of  $X_1$  or  $X_2$  is Cys. Accordingly, preferred amino acid extension include the amino acid sequences: Gly-Gly-Cys, Cys-Gly-Gly, and Cys-Gly-Cys.

25 The amino acid extension can further include one or more amino acids at either terminus of the extension. The nature of these additional amino acids is not particularly critical provided that the additional amino acids do not affect either annexin binding required for imaging or the ability of the modified annexin to chelate a radionuclide. For example, the sequences noted above can be preceded by an amino acid such as histidine and/or glycine and followed by an amino acid such as  
30 alanine.

The proposed structures for the amino acid extensions with chelated technetium are illustrated in FIGURE 1. FIGURES 1A-1C depict chemical structures for the chelation sites corresponding to the amino acid sequences Cys-Gly-Gly, Gly-Gly-Cys, and Cys-Gly-Cys, respectively. In these figures, “X” and “Y”  
35 refer to the portions of a molecule (e.g., one or more additional amino acids) beyond

the chelation site. Alternatively, "X" can represent the C-terminal and "Y" can represent the N-terminal of a molecule including the chelation site. For example, "X" can represent the sequence of amino acids leading to a polypeptide's C-terminal (e.g., annexin C-terminal) and "Y" can represent the sequence of amino acids leading to a polypeptide's N-terminal (e.g., modified annexin N-terminal). Referring to FIGURE 1A, technetium is chelated by nitrogen and sulfur atoms from the amino acid sequence Cys-Gly-Gly as shown. The figure shows that chelation includes a nitrogen atom from the amide bond of an adjacent amino acid ( $R_1$  is an amino acid substituent selected from among the substituents for naturally occurring amino acids).

The chelation site including one of the amino acid sequences noted above is located at the N-terminus of the annexin molecule. Annexin's N-terminus is remote from its biological binding site. A representative modified annexin having an amino acid sequence including the extension Cys-Gly-Gly is set forth in SEQ ID NO:2. A modified annexin having the amino acid sequence including the extension Gly-Gly-Cys is set forth in SEQ ID NO:4. A representative modified annexin having an amino acid sequence including the extension Cys-Gly-Cys is set forth in SEQ ID NO:6. The modified annexins having the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6 include their N-terminal methionine residues, which are normally removed post-translationally in both humans and *E. coli*. In preferred embodiments, the modified annexin is mutated to include serine at position 316. Such a modification results in the substitution of serine for cysteine.

The modified annexins of the present invention include an N-terminal amino acid extension. The amino acid extension can be added to the annexin through chemical means. Alternatively, in a preferred embodiment, the amino acid extension is genetically engineered (i.e., incorporated into the annexin by recombinant techniques). Accordingly, the modified annexins of the invention are preferably prepared by genetic engineering. Briefly, a cDNA encoding a wild-type or mutant annexin V was cloned into a vector (e.g., pET12a) and then transformed into an *E. coli* strain (e.g., BL21(DE3)). The cell line was then grown in growth media and the expressed protein was harvested and purified. A representative method for preparing the modified annexins of the present invention is described in Examples 1 and 2. By this method, the present invention provides bioengineered forms of annexin V having endogenous chelation sites.

In another embodiment, the present invention provides an isolated nucleic acid molecule encoding the modified annexins noted above. The present invention provides isolated nucleic acid molecules encoding modified annexins having the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.

- 5 More specifically, the present invention provides isolated nucleic acid molecules having the nucleic acid sequences set forth in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5.

- The present invention also provides a replicable expression vector comprising a nucleic acid sequence encoding a modified annexin as described above. The invention provides a replicable expression vector comprising a nucleic acid sequence encoding a modified annexin having an N-terminal chelation site that includes an amino acid sequence  $X_1$ -Gly- $X_2$ , where  $X_1$  and  $X_2$  are selected from Gly and Cys, and where at least one of  $X_1$  or  $X_2$  is Cys. The present invention also provides replicable expression vectors comprising nucleic acid sequences encoding modified annexins having the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.

- The modified annexins of the present invention are preferably purified recombinant proteins harvested from host cells incorporating expression vectors that encode the modified annexins. Thus, in another aspect, the present invention provides host cells comprising expression vectors encoding the modified annexins of the present invention. The present invention provides host cells including an expression vector comprising a nucleic acid sequence encoding a modified annexin having an N-terminal chelation site including an amino acid sequence  $X_1$ -Gly- $X_2$ , where  $X_1$  and  $X_2$  are selected from Gly and Cys, and where at least of  $X_1$  or  $X_2$  is Cys. The present invention also provides host cells including expression vectors having nucleic acid sequences encoding the modified annexins having the amino acid sequences set forth in SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.

- In another aspect, the present invention provides a modified annexin having an N-terminal chelation site to which is complexed a radionuclide. Suitable radionuclides include  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{186}\text{Re}$ ,  $^{188}\text{Re}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{94}\text{Tc}$ ,  $^{95}\text{Ru}$ ,  $^{100}\text{Pd}$ ,  $^{109}\text{Pd}$ ,  $^{212}\text{Bi}$ ,  $^{212}\text{Pb}$ , and  $^{111}\text{In}$ . In a preferred embodiment, the radionuclide complexed to the modified annexins is  $^{99\text{m}}\text{Tc}$ . A method for forming representative modified annexins chelated with technetium is described in Example 3. The radiochemical yield and radiochemical purity of the modified annexins of the present invention complexed with technetium is also provided in Example 3.

As summarized in Table 2, the radiochemical yield, defined as the percent yield of the radiolabeled protein based on the radionuclide, for the modified annexins (annexin V-116, -117, and -118) was high (i.e., 79.3, 71.0, and 94.3 percent, respectively) and comparable to the yield for the annexin-chelate conjugate (i.e.,  
5 <sup>99m</sup>Tc HYNIC-annexin V, 97.0 percent) demonstrating that the modified annexins effectively chelate technetium. Annexin V showed only insignificant nonspecific technetium binding (3.9 percent).

The mean radiochemical yield for labeling annexin V with another exogenous organic chelator (N<sub>2</sub>S<sub>2</sub>-TFP) has been reported to be 48 percent. Stratton et al.,  
10 Circulation, 92:3113-3121, 1995. The radiochemical yields provided by the modified annexins of the present invention are significantly greater than for annexin chelator conjugates.

The radiochemical purity of the technetium labeled annexins was determined after gel filtration and indicated that the modified annexins had high purity (88.3,  
15 89.3, and 98.0 percent, respectively) comparable to the annexin-chelate conjugate (99.0 percent).

The annexin-chelate conjugate used in the comparison was a hydrazinonicotinamide (HYNIC) conjugate. The conjugate and its corresponding technetium complex can be prepared by the methods described in Abrams et al., J. Nucl. Med., 31:2022-2028, 1990 and Blankenberg et al., Proc. Natl. Acad. Sci. U.S.A., 95:6349-6354, 1998. Briefly, the radiolabeled annexin conjugate is prepared  
20 in two steps: (1) reaction of annexin with succinimidyl 6-HYNIC to provide HYNIC-derivatized annexin; and (2) incubation of the derivatized annexin with <sup>99m</sup>Tc-glucosheptanoate followed by purification by gel filtration to provide the radiolabeled protein. The conjugation method noted above using a chelate-derivatized protein  
25 conjugate is characteristic for incorporating radionuclides into proteins.

Among the modified annexins of the invention, the highest radiochemical yield and radiochemical purity was obtained for annexin V-118, which includes the amino acid extension Cys-Gly-Cys. In contrast to the other modified annexins,  
30 annexin V-118 includes two cysteine residues in the amino acid extension rather than the single cysteine residue in the extensions of annexin V-116 and -117, which include the sequences Cys-Gly-Gly and Gly-Gly-Cys, respectively. Accordingly, the modified annexin including the sequence Cys-Gly-Cys can be characterized as an N<sub>2</sub>S<sub>2</sub> peptide chelate and the other modified annexins including the sequences Cys-Gly-Gly and Gly-Gly-Cys, respectively, can be characterized as N<sub>3</sub>S peptide chelates.  
35



The term " $N_xS_y$ " refers to the chelates coordination to the radionuclide. Conventional  $N_xS_y$  chelating compounds are described in international patent application Serial No. PCT/US97/12977, expressly incorporated herein by reference. In the present invention, the modified annexin including the  $N_2S_2$  peptide chelate (i.e., the amino acid extension Cys-Gly-Cys) provides the highest radiochemical yield and purity.

The bioactivity of the modified annexins of the present invention is described in Example 4. In Example 4, the affinity of the modified annexins for cell membranes was determined by their ability to compete with a fluorescein-labeled annexin V for binding to erythrocytes having exposed phosphatidylserine.

As summarized in Table 3, in the competitive binding assay, the modified annexins of the present invention exhibited a binding slightly diminished compared to the fluorescein annexin V derivative ( $IC_{50} = 9.3, 10.3,$  and  $10.1$  compared to  $6.8$  for wild-type annexin V). However, the binding of the modified annexins was substantially equivalent to the annexin chelate conjugate ( $IC_{50} = 10.1$ ).

The binding of the radiolabeled-modified annexins to erythrocytes was also determined by measuring the percent radioactivity bound to the cells after incubation with the modified annexins. The results summarized in Table 4, show that the binding of the modified annexins was comparable or greater than that for the annexin-chelate conjugate.

The results demonstrate that the modified annexins of the present invention can effectively chelate a radionuclide and retain annexin bioactivity. The results demonstrate that the modified annexins of the invention can be readily prepared in high radiochemical yield and with high radiochemical purity, and are as effective as a conventional annexin-chelate conjugate with regard to their binding properties.

In contrast to conventional annexin-chelate conjugates, the modified annexins of the invention offer the simplicity of manufacture as an advantage. While conventional chelate conjugates require multiple chemical and purification steps, the modified annexins require only incubation with the radionuclide to provide the diagnostic reagent. Thus, the present invention simply provides a clinically useful imaging reagent. Furthermore, because conventional conjugation chemistries ordinarily rely on conjugation through a protein's lysine residues, the number and location of the conjugated moiety is highly variable, and it is not uncommon for conjugation to occur at or near the site of biological activity thereby inhibiting the conjugate's biological activity. In contrast to conventional conjugation chemistries

that provide a distribution of conjugation products, the present invention provides a modified protein having a single chelation site remote from the site of biological activity.

5 In another aspect of the present invention, methods for using the modified annexins to image vascular thrombi and apoptosis are provided.

Annexin derivatives have been utilized to image vascular thrombi. The highly selective uptake of radiolabeled annexin V on left atrial thrombi in a porcine model has established the feasibility of detecting left atrial thrombi *in vivo* using gamma camera imaging of the radiolabeled annexin. Stratton et al., Circulation,  
10 92:3113-3121, 1995, expressly incorporated herein by reference. In the method, intracardiac thrombi were imaged using a  $^{99m}\text{Tc-N}_2\text{S}_2$  complex that was preformed and then covalently conjugated to annexin V. The technetium complex was prepared as described in Kasina et al., J. Nucl. Med., 32:1445-1451, 1991. To image the thrombi, the radiolabeled annexin was injected into a peripheral vein of the animal  
15 after anesthetization and left atrial thrombi formation. Gamma camera imaging provided planar and tomographic images for a period of time after injection. Autoradiographic results were obtained for select tissue sections after killing the animal. The imaging results demonstrated that left atrial appendage to blood ratios were significantly higher by both planar and tomographic methods in animals with  
20 radiolabel annexin injection and left atrial thrombi than in controls without thrombi. All thrombi were either positively or equivocally positive and the quantitative visual analysis scores and the quantitative thrombus to blood ratios were highest at 2 to 3 hours after injection.

In another aspect, the present invention provides a method for imaging  
25 vascular thrombi. The method detects the presence and location of vascular thrombi. In the method, an effective amount of a radiolabeled modified annexin of the invention is administered to a warm-blooded animal and images recorded. Preferably the images are obtained using a gamma camera and are recorded. As used herein, the term "effective amount" refers to an amount of radiolabeled modified annexin  
30 sufficient to image vascular thrombi *in vivo*. For the purposes of this invention, an effective amount of radiolabeled modified annexin is about 10 micrograms annexin per kilogram of body weight, administered intravenously.

A radiolabeled annexin derivative has also been used to image apoptosis. Localization of  $^{99m}\text{Tc}$  HYNIC-annexin V at sites of apoptotic cell death *in vivo* has  
35 been reported. For example, *in vivo* localization of  $^{99m}\text{Tc}$  HYNIC-annexin V was

observed in fulminant hepatic apoptosis induced by anti-Fas antibody rejection in BALB/c mice; acute rejection in ACI rats with transplanted heterotopic PVG cardiac allografts; and cyclophosphamide treatment of transplanted 38C13 murine B cell lymphomas. Blankenberg et al., J. Nucl. Med. 40:184-191, 1999; and Blankenberg et al., Proc. Natl. Acad. Sci. U.S.A. 95:6349-6354, 1998, each expressly incorporated herein by reference. External radionuclide imaging showed a two- to sixfold increase in the uptake of radiolabeled annexin at sites of apoptosis in all three animal models. These results demonstrate that radiolabeled annexin V can be used *in vivo* as a noninvasive means to detect and serially image tissues and organs undergoing programmed cell death.

Radiolabeled annexin has also been used for *in vivo* imaging of apoptosis during cardiac allograft rejection. Vriens et al., The Journal of Thoracic and Cardiovascular Surgery, 116:844-853, 1998, expressly incorporated herein by reference. Untreated rats served as recipients of allogenic PVG rat or syngeneic ACI rat cardiac grafts. Recipient animals underwent <sup>99m</sup>Tc HYNIC-annexin V imaging daily for seven days and region of interest analysis was used to quantify the uptake of annexin radiolabel. One animal group was treated with cyclosporin commencing on day four after transplantation. Untreated allografts showed signs of rejection four days after transplantation. Nuclear imaging indicated a significant uptake of the radiolabeled annexin in rejecting allogenic grafts than in syngeneic grafts after transplantation. A correlation between acute rejection and radiolabel uptake was observed and, after treatment with cyclosporin, no apoptotic nuclei could be identified in the allografts and radiolabel annexin uptake decreased to baseline. The results demonstrate that radiolabeled annexin V can be used *in vivo* as a noninvasive means to detect and monitor cardiac allograft rejection.

In a further aspect, the present invention also provides a method for imaging apoptosis. The method detects the presence and location of apoptotic cells. In the method, an effective amount of a radiolabeled modified annexin of the invention is administered to a warm-blooded animal and nuclear images obtained. Preferably the images are obtained using a gamma camera. As used herein, the term "effective amount" refers to an amount of radiolabeled modified annexin sufficient to image apoptotic cells *in vivo*. For the purposes of this invention, an effective amount of radiolabeled modified annexin is about 10 micrograms annexin per kilogram of body weight, administered intravenously. The method can be useful for imaging apoptosis associated with response to a chemotherapeutic agent.

The present invention provides amino acid sequences that are effective as chelation sites for a radionuclide. As noted above, the amino acid sequence includes the sequence  $X_1\text{-Gly-X}_2$ , where  $X_1$  and  $X_2$  are selected from Gly and Cys, and where at least of  $X_1$  or  $X_2$  is Cys. While the chelation site can be effectively incorporated into annexin to provide useful imaging agents as described above, it will be appreciated that the peptide chelates (i.e., the chelating amino acid sequences) can be incorporated into other molecules, including peptides and proteins, to provide modified molecules having chelation sites. The amino acid sequence can be incorporated into a molecule by chemical means. Alternatively, for peptides and proteins, the amino acid sequence is preferably incorporated by recombinant methods to provide a peptide or protein having an endogenous chelation site. The chelation site is preferably incorporated into a peptide or protein at either the C- or N-terminus, preferably distant from the peptide's or protein's binding site.

The following examples are provided for the purposes of illustration and not limitation.

## EXAMPLES

### Example 1

#### Construction and Verification of Plasmids

Four plasmids were constructed to express mutant forms of annexin V in *E. coli* under control of the phage T7 promoter. The parent expression vector was pET12a, available from Novagen Corporation (Madison, WI).

a. Construction of expression vector pJ115, encoding annexin V-115 with Cys-316->Ser mutation (position 316 refers to wild-type annexin V).

A 232-bp BstBI-BamHI restriction fragment was first isolated from plasmid pANXVC-S-N6, also known as pJ110, which encodes a modified annexin V with the Cys-316->Ser mutation. Tanaka K., et al., "Preparation and characterization of a disulfide-linked bioconjugate of annexin V with the B-chain of urokinase: an improved fibrinolytic agent targeted to phospholipid-containing thrombi," Biochemistry, 1996; 35:922-9. The same restriction fragment was then removed from plasmid pET12a-PAP1 (FIGURE 2) (Wood, B.L., et al., "Increased erythrocyte phosphatidylserine exposure in sickle cell disease: flow-cytometric measurement and clinical associations," Blood, 1996; 88:1873-80), which encodes wild-type annexin V, and the mutated fragment was ligated into the resulting vector to form plasmid pJ115. This plasmid was subjected to DNA sequence analysis to verify that the intended mutation had been correctly introduced.

b. Construction of expression vectors pJ116, pJ117, and pJ118, encoding the mutant annexin V molecules annexin V-116, annexin V-117, and annexin V-118.

For each vector to be constructed, sense and antisense oligonucleotides (sequences given in the table below) were designed to encode the desired peptide sequence with NdeI restriction sites at both ends. Equimolar amounts of sense and antisense oligonucleotide were then phosphorylated and annealed to form a double-stranded cassette with NdeI-compatible single-stranded ends. The double-stranded oligonucleotide (3.8 ng) was then ligated with plasmid pJ115 (0.42 µg) that had been previously digested with NdeI and dephosphorylated. The ligation mixture was then transformed into *E. coli* strain DH5alpha. Plasmid DNA was prepared from ampicillin-resistant colonies and screened by PCR for the presence of the desired insert. DNA sequence analysis was then performed on positive clones to verify that the desired insertion was present.

Table 1. Sense and Antisense Oligonucleotides for Construction of pJ116, pJ117 and pJ118

Name	Vector	Sequence (5' to 3')
JT-289	pJ116	TATGGCATGTGGCGGTGGCCA
JT-290	pJ116	TATGGCCACCGCCACATGCCA
JT-295	pJ117	TATGGCAGGTGGCTGTGGCCA
JT-296	pJ117	TATGGCCACAGCCACCTGCCA
JT-297	pJ118	TATGGCATGTGGCTGCGGTCA
JT-298	pJ118	TATGACCGCAGCCACATGCCA

#### Example 2

##### Expression and Purification of Proteins

Plasmids pJ116, pJ117, and pJ118 were each transformed into *E. coli* strain BL21(DE3) for cytoplasmic expression. Each resulting clone was grown overnight to saturation at 37°C with shaking in Terrific Broth containing carbenicillin (50 µg/ml). The cells were then separated from culture medium by centrifugation for 10 min at 2560 x g and washed in ice-cold buffer (50 mmol/L TrisHCl, 150 mmol/L NaCl pH 8.0). Bacteria were then disrupted by sonication in ice-cold 50 mmol/L TrisHCl pH 7.2, 10 mmol/L CaCl<sub>2</sub>, 1 mmol/L β-mercaptoethanol, and then centrifuged for 20 min at 22,530 x g. The supernatant was discarded and the annexin V bound to bacterial membranes was released by resuspending the pellet in 50 mmol/L TrisHCl pH 7.2, 20 mmol/L EDTA, 1 mmol/L β-mercaptoethanol. Bacterial

membranes were then removed by centrifugation for 20 min at 22,530 x g and the supernatant containing the annexin V was dialyzed against 20 mmol/L TrisHCl pH 8.0, 1 mmol/L  $\beta$ -mercaptoethanol. The dialysand then was applied to a Mono Q column (Pharmacia, Piscataway, NJ) and eluted with a gradient of 0 to 1 mol/L NaCl in the same buffer. The annexin V mutants all eluted at approximately 0.22 mol/L NaCl. The purified protein was concentrated by ultrafiltration to a concentration of approximately 5 mg/ml, dialyzed against 20 mmol/L HEPES pH 7.4, 100 mmol/L NaCl, and stored in aliquots at -70°C. The final yield was approximately 10 mg/L of culture, with a purity of ~98% as judged by SDS-polyacrylamide gel electrophoresis.

10

### Example 3

#### Technetium Labeling of Representative Modified Annexins

A representative procedure for technetium labeling of the modified annexins prepared as described above is as generally described in Larsen et al., "[<sup>99m</sup>Tc]Tricine: A useful precursor complex for the radiolabeling of hydrazinonicotinate protein conjugates," Bioconjugate Chem., 6:635-638, 1995, and Blankenberg et al., "In vivo detection and imaging of phosphatidylserine expression during programmed cell death," Proc. Natl. Acad. Sci. U.S.A., 95:6349-6354, 1998, both expressly incorporated herein by reference.

Briefly, annexin is first reduced with dithiothreitol and then labeled with technetium.

#### Dithiothreitol Reduction:

1. Add dithiothreitol (100 mM in water) to annexin stock solution (3-8 mg/ml in 20 mM HEPES-Na pH 7.4, 100 mM NaCl) to 1mM final concentration. For example, to 600  $\mu$ g of annexin in 200  $\mu$ l add 2  $\mu$ l of 100 mM dithiothreitol.
2. Incubate 15 min at 37°C.
3. Purify by gel filtration on Sephadex G-25 column previously equilibrated with deoxygenated 20 mM sodium citrate pH 5.2, 100 mM NaCl. Store at -70°C. For example:
  - a) Equilibrate Pharmacia NAP-5 column with three top column volumes of deoxygenated citrate buffer (20 mM sodium citrate pH 5.2, 100 mM NaCl. Deoxygenate by bubbling with argon.
  - b) Add 200  $\mu$ l of sample to column. Let run in and do not collect.
  - c) Add 500  $\mu$ l deoxygenated citrate buffer. Let run in and do not collect.

d) Add 600  $\mu$ l deoxygenated citrate buffer. Let run in and collect.  
Should now have 600  $\mu$ g in 600  $\mu$ l or 50  $\mu$ g in 50  $\mu$ l.

e) Store in aliquots of 500 to 100  $\mu$ g at  $-70^{\circ}\text{C}$ .

Technetium Labeling:

- 5        1. Thaw out 100  $\mu$ g of reduced annexin V-SH (aliquot with 50 to 100  $\mu$ g in 50  $\mu$ l of 20 mM sodium citrate pH 5.2, 100 mM NaCl).
2. Reconstitute Tin/Tricine reagent (aliquot lyophilized from 1 ml of 20 mM tricine, 80  $\mu$ g/mL  $\text{SnCl}_2 \cdot 2\text{H}_2\text{O}$ , pH 7.1) with 1 ml of distilled water.
3. Add 0.9% NaCl to annexin as necessary to bring final reaction volume  
10 to 200  $\mu$ l.
4. Add 100  $\mu$ l Tin/Tricine reagent to annexin and mix gently.
5. Add 500  $\mu$ Ci  $^{99\text{m}}\text{TcO}_4$  ( $^{99\text{m}}\text{TcO}_4$  in 0.9% NaCl) to annexin and mix gently.
6. Incubate 15 min at room temperature.
- 15       7. Determine percent incorporation of technetium into protein by ITLC in PBS (percent radiochemical yield).
8. Purify by gel filtration on Sephadex G25 column in PBS (phosphate-buffered saline). For example:
  - a) Equilibrate NAP-5 column (Sephadex G-25, Pharmacia #17-  
20 0853-01) with PBS, three top column volumes.
  - b) Add 200  $\mu$ l of sample to column. Let run in and do not collect.
  - c) Add 500  $\mu$ l PBS. Let run in and do not collect.
  - d) Add 400  $\mu$ l PBS. Let run in and collect.
- 25       9. Determine percent of technetium bound to annexin by ITLC (instant thin-layer chromatography) in PBS (percent radiochemical purity).

The results of labeling annexin V, modified annexins of the invention (identified as annexins V-116, V-117, and V-118), and an annexin chelate conjugate (HYNIC-annexin V) are summarized in Table 2.

Table 2. Annexin Labeling with Technetium: Radiochemical Yield and Purity

Protein	% Radiochemical Yield	% Radiochemical Purity
Annexin V	3.9 ± 1.6	Not applicable
Annexin V-116	79.3 ± 1.2	88.3 ± 3.2
Annexin V-117	71.0 ± 7.9	89.3 ± 4.7
Annexin V-118	94.3 ± 0.6	98.0 ± 0.0
HYNIC-annexin V	97.0%	99.0%

In the table, results given as mean ± SD with n=2 for annexin V, n=3 for mutants 116, 117, and 118, and n=1 for HYNIC-annexin V. Radiochemical purity (%) was determined after purification by gel filtration.

#### Example 4

##### Bioactivity of Representative Modified Annexins

The bioactivity of the modified annexins of the invention was determined by a competitive binding assay and by measuring binding to erythrocytes having exposed phosphatidylserine. The bioactivity of the modified annexins was compared to the activities of native annexin V and an annexin chelate conjugate, HYNIC-annexin V.

Competitive Binding Assay. The affinity of the annexins for cell membranes was determined by their ability to compete with fluorescein-labeled annexin V for binding to erythrocytes having exposed phosphatidylserine. Erythrocytes were obtained commercially (4Cplus Normal Control, Beckman-Coulter Corp., Hialeah, FL). The annexins were added at various concentrations to 1 ml of a solution containing 5 nM fluorescein-annexin V and  $8.3 \times 10^6$  erythrocytes in a buffer consisting of 10 mM HEPES-NA pH 7.4, 136 mM NaCl, 2.7 mM KCl, 5 mM glucose, 1 mg/ml BSA (buffer HNKGB) plus 2.5 mM  $\text{CaCl}_2$ . Samples were incubated for 15 min at room temperature. The cells were then centrifuged, the supernatant removed, and the fluorescein-annexin V bound to the pelleted cells was released by resuspension in 950  $\mu\text{l}$  HNKGB buffer containing 5 mM EDTA. The sample was then centrifuged again, the supernatant removed, and the concentration of fluorescein-annexin V in the supernatant determined by fluorometry. The results of this assay are summarized in Table 3 and presented as the concentration of competitor protein that inhibits 50% of the binding of fluorescein-annexin V, abbreviated  $\text{IC}_{50}$ .



**Table 3. Annexin Bioactivity by Competition Binding Assay**

Protein	IC <sub>50</sub> (nmol/L)
Annexin V	6.8 ± 0.7
Annexin V-116	9.3 ± 0.4
Annexin V-117	10.3 ± 2.5
Annexin V-118	10.1 ± 2.8
HYNIC-annexin V	10.1 ± 2.0

In the table, results given as mean ± SD with n=9 for annexin V, n=2 for mutants 116, 117, and 118, and n=9 for HYNIC-annexin V.

- 5        **Erythrocyte Binding Assay.** The ability of radiolabeled annexins to bind to cell membranes was determined by measuring the percent radioactivity bound to erythrocytes as follows. Radiolabeled annexins at 10 nmol/L final concentration was added to duplicate tubes containing a final volume of 1 ml of buffer HNKGB plus 2.5 mM CaCl<sub>2</sub>. One tube then received 4.2 x 10<sup>8</sup> erythrocytes. After a 15-min
- 10 incubation at room temperature, both tubes were centrifuged for three min at 8,320 x g. Radioactivity was then measured in 800 µl of the supernatants. The percentage of radioactivity bound to the cells was calculated using the following equation: 100(1 - (supernatant counts in presence of cells)/(supernatant counts in absence of cells)).

**Table 4. Annexin Bioactivity by Binding of Radiolabeled Proteins to Erythrocytes**

15

Protein	% Radioactivity Bound to Erythrocytes
Annexin V-116	80.3 ± 2.9
Annexin V-117	84.3 ± 2.5
Annexin V-118	86.3 ± 2.1
HYNIC-annexin V	83.9 ± 2.5

Results given as mean ± SD with n=3 for mutants 116, 117, and 118, n=7 for HYNIC-annexin V.

### Example 5

#### Hybridization of Nucleic Acid Molecules Encoding Modified Annexins to Other Nucleic Acid Sequences

20

The nucleic acid molecules of the present invention are capable of hybridizing to the nucleic acid sequences set forth in SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5, or to the complementary sequence of the nucleic acid sequences set forth in

SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5, under the following stringent hybridization conditions: incubation in 5 X SSC at 65°C for 16 hours, followed by washing under the following conditions: two washes in 2 X SSC at 18°C to 25°C for twenty minutes per wash; preferably, two washes in 2 X SSC at 18°C to 25°C for  
5 twenty minutes per wash, followed by one wash in 0.5 X SSC at 55°C for thirty minutes; most preferably, two washes in 2 X SSC at 18°C to 25°C for fifteen minutes per wash, followed by two washes in 0.2 X SSC at 65°C for twenty minutes per wash. The abbreviation "SSC" refers to a buffer used in nucleic acid hybridization solutions. One liter of the 20X (twenty times concentrate) stock SSC buffer solution  
10 (pH 7.0) contains 175.3 g sodium chloride and 88.2 g sodium citrate.

The ability of the nucleic acid molecules of the present invention to hybridize to the nucleic acid sequences set forth in SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5, or to the complementary sequence of the nucleic acid sequences set forth in SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5 can be determined utilizing the  
15 technique of hybridizing radiolabelled nucleic acid probes to nucleic acids immobilized on nitrocellulose filters or nylon membranes as set forth, for example, at pages 9.52 to 9.55 of Molecular Cloning, A Laboratory Manual (2nd edition), J. Sambrook, E.F. Fritsch and T. Maniatis eds., the cited pages of which are incorporated herein by reference.

20 In addition to the nucleic acid sequences set forth in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5, examples of representative nucleic acid sequences that encode the modified annexins of the invention and which hybridize to the complementary sequence of the nucleic acid sequences disclosed in SEQ ID NO:1, SEQ ID NO:3, and SEQ ID NO:5 can be computer generated by utilizing the  
25 degeneracy of the genetic code. Each of the representative nucleic acid sequences has a different sequence, but each encodes a modified annexin of the invention.

While the preferred embodiment of the invention has been illustrated and described, it will be appreciated that various changes can be made therein without departing from the spirit and scope of the invention.

The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. A modified annexin having an N-terminal chelation site, wherein the chelation site comprises an amino acid extension, the amino acid extension comprising a glycine and a cysteine residue.
2. The modified annexin of Claim 1 wherein the amino acid extension comprises the amino acid sequence  $X_1$ -Gly- $X_2$ , wherein  $X_1$  and  $X_2$  are selected from Gly and Cys, and wherein at least one of  $X_1$  or  $X_2$  is Cys.
3. The modified annexin of Claim 1 wherein the amino acid extension comprises the amino acid sequence Gly-Gly-Cys.
4. The modified annexin of Claim 1 wherein the amino acid extension comprises the amino acid sequence Cys-Gly-Gly.
5. The modified annexin of Claim 1 wherein the amino acid extension comprises the amino acid sequence Cys-Gly-Cys.
6. The modified annexin of Claim 1 wherein the annexin is annexin V.
7. The modified annexin of Claim 1 wherein the amino acid at position 316 of the annexin is mutated to serine.
8. The modified annexin of Claim 1 wherein the modified annexin is recombinantly formed.
9. The modified annexin of Claim 1 further comprising a radionuclide complexed to the chelation site.
10. The modified annexin of Claim 7 wherein the radionuclide is selected from the group consisting of Cu-64, Cu-67, Ga-67, Ga-68, Re-186, Re-188, Tc-99m, Tc-94, Ru-95, Pd-100, Pd-109, Bi-212, Pb-212, and In-111.
11. The modified annexin of Claim 10 wherein the radionuclide is Tc-99m.

12. A modified annexin having the amino acid sequence set forth in SEQ ID NO:2.
13. The modified annexin of Claim 12 further comprising a complexed radionuclide.
14. The modified annexin of Claim 13 wherein the radionuclide is Tc-99m.
15. A modified annexin having the amino acid sequence set forth in SEQ ID NO:4.
16. The modified annexin of Claim 15 further comprising a complexed radionuclide.
17. The modified annexin of Claim 16 wherein the radionuclide is Tc-99m.
18. A modified annexin having the amino acid sequence set forth in SEQ ID NO:6.
19. The modified annexin of Claim 18 further comprising a complexed radionuclide.
20. The modified annexin of Claim 19 wherein the radionuclide is Tc-99m.
21. An isolated nucleic acid molecule encoding a modified annexin having an N-terminal chelation site, wherein the chelation site comprises an amino acid extension, the amino acid extension comprising a glycine and a cysteine residue.
22. An isolated nucleic acid molecule encoding a modified annexin having the amino acid sequence set forth in SEQ ID NO:2.
23. An isolated nucleic acid molecule encoding a modified annexin having the amino acid sequence set forth in SEQ ID NO:4.
24. An isolated nucleic acid molecule encoding a modified annexin having the amino acid sequence set forth in SEQ ID NO:6.

25. An isolated nucleic acid molecule having the nucleic acid sequence set forth in SEQ ID NO:1.

26. An isolated nucleic acid molecule having the nucleic acid sequence set forth in SEQ ID NO:3.

27. An isolated nucleic acid molecule having the nucleic acid sequence set forth in SEQ ID NO:5.

28. A replicable expression vector comprising a nucleic acid sequence encoding a modified annexin having an N-terminal chelation site comprising an amino acid sequence  $X_1$ -Gly- $X_2$ , wherein  $X_1$  and  $X_2$  are selected from Gly and Cys, and wherein at least one of  $X_1$  or  $X_2$  is Cys.

29. A replication expression vector of Claim 28 comprising a nucleic acid sequence encoding a modified annexin having the amino acid sequence set forth in SEQ ID NO:2.

30. A replication expression vector of Claim 28 comprising a nucleic acid sequence encoding a modified annexin having the amino acid sequence set forth in SEQ ID NO:4.

31. A replication expression vector of Claim 28 comprising a nucleic acid sequence encoding a modified annexin having the amino acid sequence set forth in SEQ ID NO:6.

32. A host cell comprising a vector of Claim 28.

33. A host cell comprising a vector of Claim 29.

34. A host cell comprising a vector of Claim 30.

35. A host cell comprising a vector of Claim 31.

36. An isolated nucleic acid molecule that is capable of hybridizing to the nucleic acid sequence set forth in SEQ ID NO:1, or to the complementary sequence of the nucleic acid molecule set forth in SEQ ID NO:1, under stringent hybridization conditions.

37. An isolated nucleic acid molecule that is capable of hybridizing to the nucleic acid sequence set forth in SEQ ID NO:3, or to the complementary sequence of the nucleic acid molecule set forth in SEQ ID NO:3, under stringent hybridization conditions.

38. An isolated nucleic acid molecule that is capable of hybridizing to the nucleic acid sequence set forth in SEQ ID NO:5, or to the complementary sequence of the nucleic acid molecule set forth in SEQ ID NO:5, under stringent hybridization conditions.

39. A chelation moiety comprising the amino acid sequence  $X_1$ -Gly- $X_2$ , wherein  $X_1$  and  $X_2$  are selected from Gly and Cys, and wherein at least one of  $X_1$  or  $X_2$  is Cys.

40. A chelation moiety comprising the amino acid sequence Gly-Gly-Cys.

41. A chelation moiety comprising the amino acid sequence Cys-Gly-Gly.

42. A chelation moiety comprising the amino acid sequence Cys-Gly-Cys.

43. A modified protein having a chelation site comprising the amino acid sequence  $X_1$ -Gly- $X_2$ , wherein  $X_1$  and  $X_2$  are selected from Gly and Cys, and wherein at least one of  $X_1$  or  $X_2$  is Cys.

44. The modified protein of Claim 43 further comprising a radionuclide complexed to the chelation site.

45. The modified protein of Claim 43 wherein the chelation site comprises the amino acid sequence Gly-Gly-Cys.

46. The modified protein of Claim 45 further comprising a radionuclide complexed to the chelation site.

47. The modified protein of Claim 43 wherein the chelation site comprises the amino acid sequence Cys-Gly-Gly.

48. The modified protein of Claim 47 further comprising a radionuclide complexed to the chelation site.

49. The modified protein of Claim 43 wherein the chelation site comprises the amino acid sequence Cys-Gly-Cys.

50. The modified protein of Claim 49 further comprising a radionuclide complexed to the chelation site.

51. A method for imaging vascular thrombi comprising:

administering to a warm-blooded animal an effective amount a modified annexin having an N-terminal chelation site, wherein the chelation site comprises an amino acid extension, the amino acid extension comprising a glycine and a cysteine residue, and wherein a radionuclide is complexed to the chelation site; and

obtaining nuclear images of the animal to determine the presence and location of vascular thrombi.

52. The method of Claim 51 wherein the amino acid extension comprises the amino acid sequence  $X_1$ -Gly- $X_2$ , wherein  $X_1$  and  $X_2$  are selected from Gly and Cys, and wherein at least one of  $X_1$  or  $X_2$  is Cys.

53. The method of Claim 51 wherein the amino acid extension comprises the amino acid sequence Gly-Gly-Cys.

54. The method of Claim 51 wherein the amino acid extension comprises the amino acid sequence Cys-Gly-Gly.

55. The method of Claim 51 wherein the amino acid extension comprises the amino acid sequence Cys-Gly-Cys.

56. A method for imaging apoptosis comprising:

administering to a warm-blooded animal an effective amount a modified annexin having an N-terminal chelation site, wherein the chelation site comprises an amino acid extension, the amino acid extension comprising a glycine and a cysteine residue, and wherein a radionuclide is complexed to the chelation site; and

obtaining nuclear images of the animal to determine the presence and location of apoptotic cells.

57. The method of Claim 56 wherein the amino acid extension comprises the amino acid sequence  $X_1$ -Gly- $X_2$ , wherein  $X_1$  and  $X_2$  are selected from Gly and Cys, and wherein at least one of  $X_1$  or  $X_2$  is Cys.

58. The method of Claim 56 wherein the amino acid extension comprises the amino acid sequence Gly-Gly-Cys.

59. The method of Claim 56 wherein the amino acid extension comprises the amino acid sequence Cys-Gly-Gly.

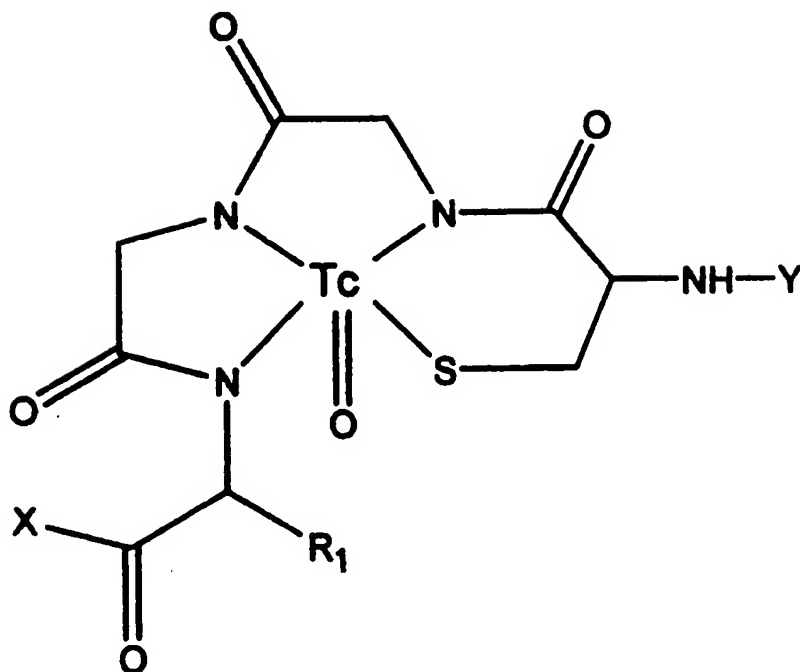
60. The method of Claim 56 wherein the amino acid extension comprises the amino acid sequence Cys-Gly-Cys.

61. The method of Claim 56 wherein the apoptosis is associated with rejection as a result of transplantation.

62. The method of Claim 56 wherein the apoptosis is associated with response to a chemotherapeutic agent.



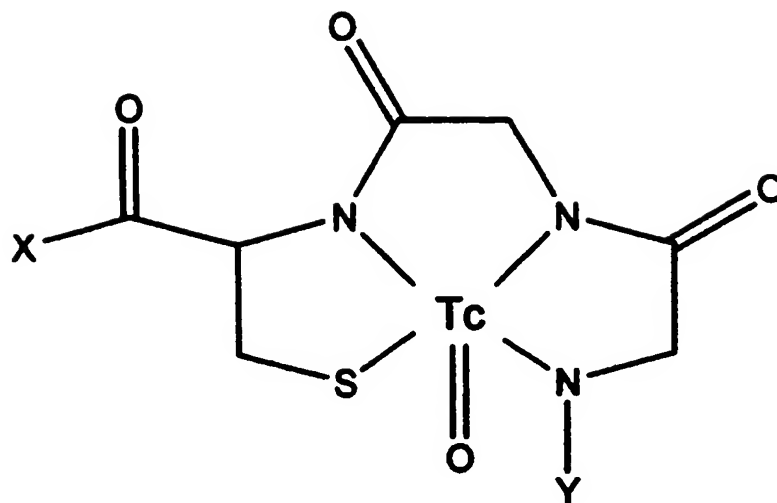
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(NH<sub>2</sub>) Cys-Gly-Gly (CO<sub>2</sub>H)

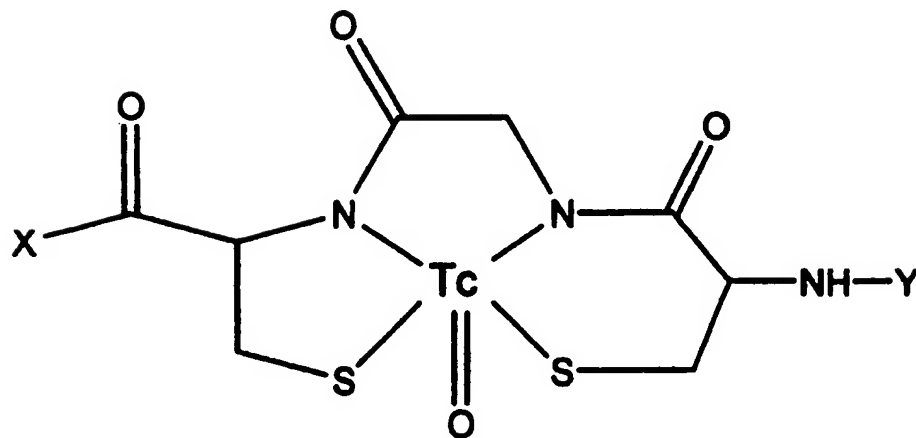
*Fig. 1A.*

2/3



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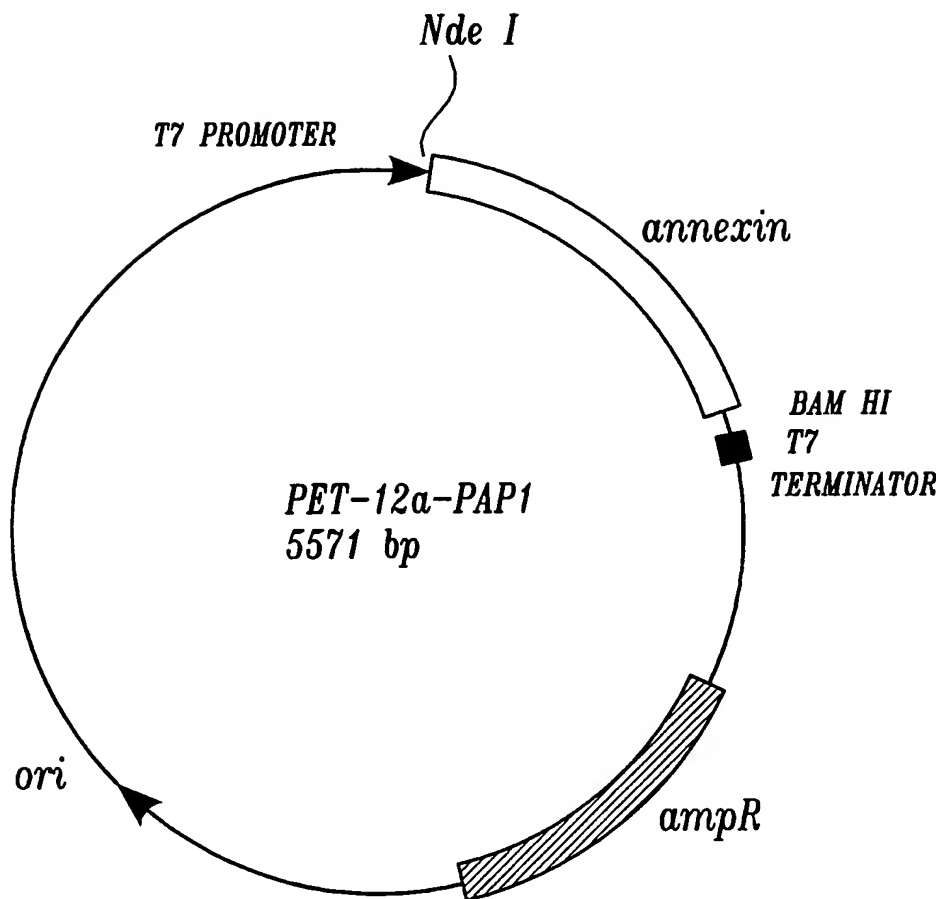
*Fig. 1 B.*



(NH<sub>2</sub>) Cys-Gly-Cys (CO<sub>2</sub>H)

*Fig. 1 C.*

3/3



*Fig. 2.*

## SEQUENCE LISTING

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Brown, David S.

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 50                                      55                                      60  
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 115                                      120                                      125  
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Lys Trp Gly Thr Asp Glu Glu Lys Phe Ile Thr Ile Phe Gly Thr Arg  
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Ser Val Ser His Leu Arg Lys Val Phe Asp Lys Tyr Met Thr Ile Ser  
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Glu Gln Leu Leu Leu Ala Val Val Lys Ser Ile Arg Ser Ile Pro Ala  
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Leu	Arg	Ala	Ile	Lys	Gln	Val	Tyr	Glu	Glu	Glu	Tyr	Gly	Ser	Ser	Leu	
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Glu	Asp	Asp	Val	Val	Gly	Asp	Thr	Ser	Gly	Tyr	Tyr	Gln	Arg	Met	Leu	
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Leu Leu Ser Gly Glu Asp Asp  
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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US00/14324

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(7) :C07K 14/00, 1/00, 17/00

US CL :530/350; 536/23.1; 514/12; 435/320.1

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 530/350; 536/23.1; 514/12; 435/320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EAST

search terms: annexin

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	VRIENS, P. W. "The Use of Technetium TC 99M Annexin V for In Vivo Imaging of Apoptosis During Cardiac Allograft Rejection," Journal of Thoracic and Cardiovascular Surgery. November 1998, Vol. 116, No. 5, pages 844-853.	1-62

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&" document member of the same patent family
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

31 JULY 2000

Date of mailing of the international search report

19 SEP 2000

Name and mailing address of the ISA/US  
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